Selective Protein Adduct Formation of Diclofenac Glucuronide Is Critically Dependent on the Rat Canalicular Conjugate Export Pump (Mrp2)†

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Previous work demonstrates that the reactive acyl glucuronide of the nonsteroidal antiinflammatory drug diclofenac forms selective protein adducts in the liver, which may play a causal role in the pathogenesis of diclofenac-associated liver toxicity. Because glucuronide conjugates can be exported into the bile, we explored the role of diclofenac glucuronide hepatobiliary transport in the formation of site-specific protein adducts. Specifically, to analyze intracellular (hepatocytes) versus extracellular (biliary tree) targeting of proteins, we have compared the pattern of diclofenac binding in normal Wistar rats with that in mutant transport-deficient (TR−) rats which lack the functional canalicular isoform of the conjugate export pump, Mrp2. In bile duct-cannulated normal rats, > 50% of an iv injected dose of [14C]diclofenac appeared in the bile over a 90-min period. In contrast, in TR− rats virtually no hepatobiliary excretion of diclofenac glucuronide was found. After administration of diclofenac (30 mg/kg/day, ip for 3 days) to rats of both genotypes, a major protein adduct of an apparent molecular mass of 118 kDa was selectively detected by immunoblotting in isolated canalicular, but not in basolateral, membrane subfractions of wild-type rats, whereas no plasma membrane adducts could be found in the livers of TR− rats. Furthermore, immunohistochemical analysis using an anti-diclofenac antibody revealed the presence of distinct diclofenac-modified proteins on canalicular membranes of liver sections from diclofenac-treated normal rats, whereas no adducts could be identified in livers of TR− rats. In Western blots, the major diclofenac-modified canalicular membrane protein did not comigrate with Mrp2, indicating that the glucuronide carrier itself was unlikely to be a target. Collectively, the results demonstrate that the reactive diclofenac glucuronide is selectively transported into bile via Mrp2 and that hepatobiliary transport is critical for diclofenac covalent binding to proteins in the biliary tree.

Introduction

Diclofenac and other nonsteroidal antiinflammatory drugs (NSAIDs) have been associated with mild hepatic injury (release of aminotransferases) in a small fraction of recipients and also with rare cases of fulminant injury (release of aminotransferases) in a small fraction of hepatocellular target proteins. Reactive species that covalently interact with amines in the liver and that the resulting acyl glucuronides are reactive species that covalently interact with a number of hepatocellular target proteins. Among these alkylated proteins, a canalicular plasma membrane protein has recently been identified as dipeptidyl peptidase IV (DPP IV). The nature of other molecular targets in the liver has not yet been elucidated. In addition, a causal role of the covalently modified proteins in the pathogenesis of diclofenac-associated liver injury has not yet been convincingly demonstrated.

Glucuronide conjugates, depending on their reactivity, can covalently bind to proteins within hepatocytes. However, glucuronides are also likely to be secreted into the bile across the canalicular plasma membrane, where they could react with targets in the biliary tree. Hepatobiliary transport of drug conjugates normally occurs by ATP-dependent transport, which is mediated by the multispecific conjugate export pump (canalicular organic anion transporter, cMOAT) (14, 15). Recently, this carrier has been cloned (16, 17), and its homology to the human multidrug resistance-associated protein, MRP (18), has been demonstrated. In the rat, the canalicular isoform of this transporter is called Mrp2 (17, 19) or Mrp (20). A direct transport function of Mrp2 for glutathione conjugates and leukotriene C4 has recently been demonstrated by expression of the carrier in COS-7 cells and in Xenopus laevis oocytes (21). However, it has remained speculative whether this carrier is involved in the trans-
membrane transport of NSAID glucuron conjugates including diclofenac. In addition, the role of Mrp2 as a potential target of covalent modification by the reactive diclofenac glucuronide has not been addressed.

To further explore the role of Mrp2-mediated canalicular transport on the biliary excretion and canalicular binding of diclofenac, we have used a mutant transport-deficient (TR−) rat strain. These rats, originally derived from the Wistar strain, are hyperbilirubinemic due to a lack of canalicular export of bilirubin diglucuronide. Specifically, they do not express the canalicular conjugate transport protein adduct formation in normal and Mrp2-deficient rats. Here we demonstrate that TR− rats are unable to export this metabolite into bile and that covariant adduct formation to canalicular membrane proteins is critically dependent on a functional Mrp2 protein.

Experimental Procedures

Materials. Diclofenac sodium was purchased from Sigma Chemical Co. (St. Louis, MO). [phenylacetic acid ring-U-14C]-Diclofenac (2.37 × 1011 Bq/mol; radiochemical purity, ~98%) was generously provided by Novartis, Ltd. (Basel, Switzerland). Horseradish peroxidase-conjugated anti-rabbit IgG and hyperfilms were from Amersham (Buckinghamshire, England). The monoclonal mouse anti-rat DPP IV antibody (CLB 4/40) was kindly provided by Dr. B. Stieger, Department of Clinical Pharmacology and Toxicology, University Hospital, Zurich. The affinity-purified anti-diclofenac IgG was prepared as previously described (10). The mouse monoclonal anti-rat Mrp2 antibody (M211-5) was prepared as described (17). All other chemicals were obtained from Sigma and were of the highest grade available.

Animals and Treatment. Permission for all animal studies was obtained from the local authorities, and all study protocols were in compliance with institutional guidelines. Male Wistar rats and transport mutant (TR−) rats (derived from Wistar) were either from the breeding colony at the Department of Gastrointestinal and Liver Diseases, Academic Medical Center, Amsterdam, or from the breeding colony at the University Hospital, Zurich. The rats were adapted to the laboratory conditions for at least 10 days and were 10 weeks of age at the time of the experiments. They received Nafag 890 rat pellets (Nafag, Gossau, Switzerland) and water ad libitum. The animals were housed in groups of five in Macrolone cages with wood shavings as bedding, under controlled environmental conditions. Diclofenac sodium was administered by intraperitoneal injection (30 mg/kg/d) for 3 consecutive days. Control rats received saline only. Twenty-four hours after the last injection, the rats were euthanized and the liver was quickly excised and used for the isolation of plasma membranes. For immunohistochemistry, pieces of various liver lobes were fixed in 4% buffered formalin for 12 h, processed, and embedded in paraffin.

Bile Duct Cannulation and Quantification of [14C]-Diclofenac Excretion. Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (65 mg/kg, in saline). Following laparotomy, the common bile duct was exposed and cannulated approximately 1 cm distal to the bifurcation with PE tubing (0.58-mm i.d. and 0.96-mm o.d. for Wistar rats, 0.28-mm i.d. and 0.61-mm o.d. for TR− rats; Portex Ltd., Hythe, U.K.). Body temperature was monitored with a rectal thermometer and kept at 37°C using a heating pad. When the bile flow was constant, usually after 5 min, diclofenac (3 mg/kg of body wt, dissolved in saline), containing trace amounts of [14C]-diclofenac, was injected into the vena cava inferior. Bile samples were collected in preweighed Eppendorf tubes at 5–10-min intervals and kept on ice. The sample volume was determined gravimetrically, assuming that the specific weight of bile was 1. Aliquots of bile or urine samples (collected from urinary bladder puncture) were transferred to scintillation vials. Following addition of scintillation cocktail (Ultima Gold, Packard), the radioactivity was counted in a Beckman LS 6000 LL liquid scintillation counter.

HPLC Analysis of Microsomal Diclofenac Glucuronide Formation. Liver microsomes from Wistar and TR− rats were prepared as previously described (11). Protein content was determined (24), and the samples were stored at −80°C. Rates of UGT-catalyzed diclofenac acyl glucuronide formation were determined in microsomal suspensions (25–27). Briefly, 3 mg of microsomal protein was preincubated in 1 mL of 100 mM sodium acetate buffer, pH 6.0, containing 7.5 mM MgCl2, 5 mM D-saccharic acid 1,4-lactone, 0.1 mg/mL Brij 58, and 2 mM uridine 5′-diphospho-N-acetylglucosamine for 20 min on ice in order to activate UGT. The temperature was then shifted to 37°C for 2 min, and the reaction was started by the addition of uridine 5′-diphosphoglucuronic acid (final concentration 3.5 mM) and diclofenac (final concentration 5 mM). Samples were terminated in saline, analyzed after 5, 10, 20, and 60 min. To quantitate the glucuronon conjugate (but not free diclofenac), the reaction was stopped by the addition of ice-cold trichloroacetic acid (final concentration 5%, v/v). To precipitate the protein, the reaction vials were kept on ice for 20 min and centrifuged at 15000g, at 4°C for 10 min. The supernatants were buffered to pH 3–6 with some sodium bicarbonate crystals and were mixed 1:1 with 36 mM tetraethylamine/acetic acid, pH 5.0. Twenty microcrystals thereof were injected onto the HPLC column. Rates of microsomal glucuronide formation were calculated from the slope of the time curve run under linear conditions.

HPLC analysis of diclofenac was carried out on a HP 1090 liquid chromatograph system (Hewlett-Packard, Switzerland) using a reversed-phase Nucleosil 120 C18 column and a UV detection system set at 280 nm. The starting mobile phase consisted of water—3.6 mM tetraethylamine/acetic acid, pH 3.7—methanol (30:20:50, v/v/v). Elution was performed at a flow rate of 1 mL/min using two linear gradients for 15 min each. After the first 15 min the mobile phase consisted of water—3.6 mM tetraethylamine/acetic acid, pH 3.7—methanol (20:20:60, v/v/v), and at the end of the run it consisted of 100% methanol. The retention times of diclofenac glucuronide and free diclofenac were 4.4 and 16.0 min, respectively. The diclofenac glucuronide peak was absent when incubations were run without UDP-glucuronic acid. In addition, control experiments were run in which the samples, following incubation with microsomes and substrates, were divided into two aliquots. One aliquot was directly injected onto the HPLC column to determine free diclofenac. The second aliquot was incubated at pH 5.0 with 2500 units of bovine liver β-glucuronidase at 37°C for 60 min. Twenty microcrystals of the glucuronidase-treated samples was then injected onto the column. The amount of diclofenac glucuronide was calculated by subtracting the amount of free diclofenac in the untreated sample from that in the glucuronidase-treated sample and yielded similar amounts of glucuronide as those directly calculated from the diclofenac glucuronide peak.

Isolation and Characterization of CLPM and bLPM. Subcellular fractions enriched in canalicular (CLPM) and basolateral (bLPM) plasma membranes were prepared according to Meier et al. (28). The degree of purification was determined with the marker enzyme activities Na+/K+/ATPase, Mg2+-ATPase (29), and leucine aminopeptidase (30). Total protein was determined according to the method of Bradford (24), using BSA as standard protein.

SDS–PAGE and Immunoblotting. Protein samples (50 μg/lane) were mixed with sample buffer and solubilized by boiling for 5 min (31). A BioRad Mini-Protein II cell with 4%
stacking and 8% resolving gels was used. Before loading the samples, all gels were prerun for 20 min at 30 mA/gel. Electrophoresis was typically run at 30 mA/gel at room temperature for approximately 75 min. The resolved proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) at 200 V in transfer buffer (25 mM Tris, 192 mM glycine, 20% [v/v] methanol) for 1 h at 4°C.

Immunodetection of diclofenac adducts was performed as previously described (32). Briefly, the nitrocellulose membranes were blocked overnight in 154 mM NaCl, 10 mM Tris-HCl, pH 7.6, 2.5% (w/v) casein overnight at 4°C. All subsequent steps were carried out at room temperature under continuous shaking. The blocked membranes were incubated with anti-diclofenac antibody diluted in wash buffer (154 mM NaCl, 10 mM Tris-HCl, pH 7.6, 0.5% [w/v] casein) for 3 h. Nonbound antibody was removed by two 10-min washes with wash buffer. The blots were then incubated for 2 h with horseradish peroxidase-coupled anti-rabbit IgG diluted in wash buffer and subsequently washed with wash buffer for 4×10 min and with 200 mM NaCl, 50 mM Tris-HCl, pH 7.4, for another 4×10 min.

For the detection of all other proteins, the following protocol was used. The nitrocellulose membranes were blocked in blocking buffer [PBS, 0.25% (v/v) Tween-20, 5% (w/v) nonfat milk powder] overnight at 4°C. All subsequent steps were carried out at room temperature under continuous shaking. The blocked membranes were incubated with the primary antibody diluted in blocking buffer for 1 h. Nonbound antibody was removed by washing once for 15 min and then twice for 5 min in wash buffer (PBS, 0.25% Tween-20). The blots were then incubated for 1 h with horseradish peroxidase-coupled secondary antibody diluted in blocking buffer and subsequently washed once for 15 min and twice for 5 min with wash buffer.

All blots were finally developed using SuperSignal ECL reagents (Pierce, Rockford, IL) according to the manufacturer’s instructions and exposed to Hyperfilm ECL. Digital images were acquired using the Kodak DCS 460 digital camera system.

**Immunofluorescence and Confocal Laser Scanning Microscopy.** Sections (6 μm thick) were prepared from paraffin-embedded tissues and mounted on glass slides. The sections were deparaffinized with xylene and a descending ethanol series. After a rinse in PBS, the slides were incubated with anti-diclofenac antibody diluted in PBS/0.5% BSA for 1 h at room temperature. Nonbound antibody was washed away with PBS, and the sections were incubated with Cy3-labeled goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) diluted in PBS/0.5% BSA for 1 h at room temperature. After rinsing in PBS, the slides were mounted in Immuno-mount (Shandon, Pittsburgh, PA) and examined with a Zeiss LSM 410 confocal laser scanning microscope.

**Results**

**Biliary Excretion of Diclofenac Acyl Glucuronide.** It has been previously demonstrated that diclofenac is extensively metabolized in the liver and that, in rats, the major metabolite is the acyl glucuronide, which is excreted into bile (33). Here, 57% of an intravenously injected dose of [14C]diclofenac (3 mg/kg) appeared in the bile of bile duct-cannulated Wistar rats during the first 90 min postadministration (Figure 1). The rate of hepatobiliary excretion was highest at 30 min and declined thereafter. To specifically define the role of Mrp2 in the hepatobiliary transport of diclofenac metabolites, the biliary excretion of [14C]diclofenac was studied in Mrp2-deficient TR− rats. In contrast to wild-type rats, the radioactivity excreted into bile of bile duct-cannulated TR− rats was only a small fraction of that in Wistar rats, amounting to <2% of the total injected dose (Figure 1). Furthermore, the maximal rate of biliary excretion was attained between 60 and 80 min post-administration, suggesting prolonged hepatic retention of diclofenac metabolites in the transport-deficient rats. These findings demonstrate that TR− rats exhibit a greatly reduced transport capacity for diclofenac into bile. Moreover, the data imply that the conjugate export pump Mrp2 is primarily involved in mediating the transmembrane transport of diclofenac acyl glucuronide into bile.

To ascertain whether the reason for the observed low biliary export of diclofenac was not a compromised ability to form glucuronide conjugates in the TR− rat, we next compared the rates of hepatic microsomal diclofenac glucuronidation in the two genotypes (Table 1). The data demonstrate that diclofenac UGT activity in TR− rats did not exhibit a deficit in activity; in fact, the enzyme activity was 1.7-fold higher than that in Wistar rats.

**Role of Mrp2 in the Covalent Binding of Diclofenac to Hepatic Canalicular Plasma Membrane Protein.** To explore whether the presence of a functional canalicular glucuronide export pump may determine the qualitative and/or quantitative pattern of diclofenac covalent binding in the liver, the amount and subcellular distribution of diclofenac-modified proteins was studied by immunoblotting. First, probing with anti-diclofenac antibody of whole-liver homogenates derived from di-
enzymes, were prepared from both TR and Wistar rats were injected intraperitoneally with diclofenac sodium (30 mg/kg/day for 3 consecutive days) or saline. Twenty-four hours after the last dose, the rats were euthanized and the livers removed and homogenized. Total protein from diclofenac-treated rats was resolved by SDS–PAGE (50 μg/lane), transferred to nitrocellulose, probed with anti-diclofenac antibody, and developed with a HRP-coupled goat anti-rabbit antibody using ECL detection. The Western blot demonstrates the presence of a major protein band of an apparent molecular mass of 118 kDa (arrow) in the liver of Wistar rats (W) but not in that of TR rats. On the left, the migration pattern of the marker proteins in the SDS–PAGE gel is indicated.

diclofenac-treated normal Wistar rats, obtained 24 h after a 3-day treatment with diclofenac (30 mg/kg/day), revealed a major protein band of an apparent molecular mass of 118 kDa (Figure 2), which was absent in livers of saline controls. In addition, nonspecific epitopes were recognized in liver homogenates of both treated and nontreated rats. Importantly, the 118-kDa adduct was not present in liver homogenates of TR rats treated with diclofenac, nor were other treatment-related adducts conspicuous.

Next, we more specifically analyzed the pattern of adduct formation to plasma membrane proteins because increasing evidence indicates that hepatocellular plasma membrane proteins constitute a major subcellular target of diclofenac covalent binding (9, 10, 13, 32). Therefore, two different plasma membrane subfractions, enriched in either cLPM or bLPM and characterized by marker enzymes, were prepared from both TR and Wistar rats pretreated with diclofenac (Table 2). The data show that the degree of enrichment of plasma membranes during the purification procedure was high (Na+,K+-ATPase for both cLPM and bLPM). In addition, the degree of purification of the specific canalicular subdomain was also satisfactory, as judged from the selective enrichment of the cLPM markers, Mg2+-ATPase and leucine aminopeptidase, and it was similar in both rat genotypes (even higher in the TR rats). Accordingly, the proteins from these plasma membrane subfractions were resolved and probed with anti-diclofenac antibody. In Western blots, a major protein adduct with an apparent molecular mass of 118 kDa was prominent (Figure 3). In normal Wistar rats, this antigen was present in cLPM only and could not be detected in bLPM. In TR rats, the 118-kDa adduct was not detectable in either cLPM or bLPM. Because the only known genetic difference between Wistar and TR rats is the presence or absence of a functional Mrp2 protein, these results point to a critical role of the canalicular Mrp2 transporter for diclofenac covalent binding.
Diclofenac Covalent Binding and Mrp2


Table 2. Characterization of Isolated cLPM and bLPM with Enzyme Markers

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>Wistar rats</th>
<th>TR- rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>liver</td>
<td>cLPM</td>
</tr>
<tr>
<td>Na+,K+-ATPase</td>
<td>19 (1)</td>
<td>372 (×15)</td>
</tr>
<tr>
<td>Mg2+-ATPase</td>
<td>57 (1)</td>
<td>433 (×8)</td>
</tr>
<tr>
<td>Leucine aminopeptidase</td>
<td>7 (1)</td>
<td>34 (×5)</td>
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a The data represent enzyme activities obtained from a representative plasma membrane preparation. The numbers in parentheses indicate the fold enrichment over whole-liver homogenates.

Discussion

This study was designed to explore the role of hepatobiliary excretion of diclofenac acyl glucuronide, the major protein-reactive metabolite of diclofenac in the rat, in interacting with and covalently binding to hepatic proteins. The results demonstrate that the hepatic canalicular isoform of the conjugate export pump, which mediates the transport of diclofenac glucuronide across the canalicular plasma membrane into bile, plays a role which is critical for covalent adduct formation. Specifically, this transmembrane carrier is necessary for binding to occur, which implies that covalent interactions between diclofenac glucuronide and proteins take place in the canalicular lumen and more distally in the biliary tree, rather than within the hepatocytes.

These conclusions were derived from two major observations. First, TR- rats, which lack a functional Mrp2 protein, were not able to excrete diclofenac glucuronide into bile, whereas the wild-type (Wistar) counterpart transported more than 50% of an intravenously administered dose of diclofenac into bile. Second, as a consequence, TR- rats did not exhibit adducts to liver plasma membrane proteins following a treatment schedule with diclofenac that provoked massive covalent binding in normal Wistar rats. This apparent absence of diclofenac-modified proteins is unlikely to be due to reduced formation of the reactive ester glucuronide in the mutant rats because TR- rats did not exhibit defects in diclofenac glucuronosyltransferase activity. On the other hand, one could argue that the lack of protein adducts in TR- rats could reflect alterations in the expression of the diclofenac-binding protein(s) in the liver. However, the canalicular form of DPP IV, which is a major target for diclofenac binding (13), is equally present in both rat phenotypes, as shown by immunochemical analysis (Figure 4). Finally, to explain the absence of protein adducts in the TR- rats, one could presume that the glucuronide export pump itself might be alkylated by diclofenac glucuronide. However, as judged from the different migration patterns of the diclofenac adduct and Mrp2, the latter was unlikely to be a target (Figure 4). Instead,
The fact that we have found increased levels of radioactive glucuronide export into the sinusoids, but its export pump, which has been described to be present on the basolateral membrane, is selectively absent in rats that were intravenously administered [14C]diclofenac (approximately 9-fold higher than in Wistar controls at 90 min postinjection) lends further support to the concept that in those rats where hepatobiliary excretion is compromised, the majority of diclofenac metabolites are excreted via the urinary pathway. This model may also explain why the canalicular membrane proteins are selectively alkylated by diclofenac glucuronide in contrast to other cellular components. The energy-driven extrusion by Mrp2 generates a concentration of diclofenac glucuronide in the canalicular lumen that is much higher than that within the hepatocyte or in the blood. This high intracanalicular concentration strongly stimulates alkylation by pushing the reaction toward the alkylated product.

The role of hepatobiliary excretion and covalent binding of reactive diclofenac glucuronide in the pathogenesis of diclofenac-associated liver dysfunction and toxicity is still poorly defined, mainly because an animal model for diclofenac hepatitis is not available. Nevertheless, the finding that canalicular transport is critical for binding of the acyl glucuronides to target proteins raises the question of the significance of these adducts in the biliary tree. Interestingly, the transport-deficient TR- rat has been used as a model for the Dubin–Johnson syndrome in humans which is characterized by an inherited defect in the secretion of amphiphilic anionic conjugates from hepatocytes into the bile (14). Recently, molecular analysis of the disease has revealed that, analogously to the TR- rat, the canalicular isoform of the MRP is selectively absent (35). Whether these transport-deficient patients exhibit less frequent adverse effects of NSAIDs as the rest of the susceptible population has, however, not been determined. Such data will not be easily obtained as the Dubin–Johnson syndrome is very rare. Although the overall relationship between altered glucuronide export into bile and the precipitation of NSAID-induced liver injury is not established, acyl glucuronides eventually reach the gastrointestinal tract, where these protein-reactive metabolites may be directly or indirectly involved in the intestinal adverse effects associated with many of the NSAIDs including diclofenac.

In conclusion, this study provides evidence that diclofenac glucuronide is excreted into bile via Mrp2 and that a functional Mrp2 protein, and hence hepatobiliary excretion, is necessary for diclofenac glucuronide to covalently bind to canalicular proteins. These findings could have wider implications for a better understanding of the pathogenesis of diclofenac-associated adverse effects in the liver.

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References


It seems as though the targeted protein(s) were canalicular proteins facing the lumen of the bile canaliculus and that the diclofenac glucuronide may attack nuleophilic groups of amino acid residues after being expelled from the hepatocyte into the biliary tree. This is in accordance with previous findings that DPP IV is primarily located at the extracellular (luminal) side of the cell (34).

The presence of a predominant 118-kDa plasma membrane protein adduct in this study seems to contrast with previously published work in which we have found a different adduct pattern (10). In the latter study, however, livers were analyzed 2 h after the last injection of diclofenac, while in this study the protein adducts were resolved 24 h posttreatment. It is known that adduct patterns may disappear or shift over time as a function of their half-lives and/or subcellular distribution, stronger protein bands becoming less prominent and vice versa (9, 10).

On the basis of our observations, we propose a model of how covalent adduct formation to hepatic canalicular proteins may occur (Figure 6). In normal hepatocytes (Figure 6A), diclofenac is converted to a number of metabolites that are exported across the basolateral membrane into the bloodstream. The acyl glucuronide of diclofenac, however, is transported across the canalicular plasma membrane into the bile via Mrp2. In the biliary tree, the reactive intermediate may covalently react with target proteins facing the extracellular plasma membrane domain. In contrast, in the TR- phenotype, the acyl glucuronide cannot be transported into bile. Instead, the glucuronide will be exported across the basolateral plasma membrane (Figure 6B). Alternatively, a quantitatively minor isoform of the conjugate export pump, which has been described to be present on the lateral membrane of hepatocytes (16), could participate in glucuronide export into the sinusoids, but its overall contribution has not been examined. In addition, the fact that we have found increased levels of radioactivity in the urine of TR- rats that were intravenously administered [14C]diclofenac (approximately 9-fold higher concentrations of radioactivity of urinary bladder content over Wistar controls at 90 min postinjection) lends further support to the concept that in those rats where biliary elimination is compromised, the majority of diclofenac metabolites are excreted via the urinary pathway. This model may also explain why the canalicular membrane proteins are selectively alkylated by diclofenac glucuronide in contrast to other cellular components. The energy-driven extrusion by Mrp2 generates a concentration of diclofenac glucuronide in the canalicular lumen that is much higher than that within the hepatocyte or in the blood. This high intracanalicular concentration strongly stimulates alkylation by pushing the reaction toward the alkylated product.

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Diclofenac Covalent Binding and Mrp2


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